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Onecut1 and Onecut2 transcription factors operate downstream of Pax6 to regulate horizontal cell development

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ABSTRACT

Genetic studies of the last decades strongly indicated that generation of particular retinal cell types is governed by gene regulatory networks of transcription factors and their target genes. The paired and homeodomain transcription factor Pax6 plays a pivotal role in retinal development as its inactivation in the retinal progenitor cell population leads to abolished differentiation of all retinal cell types. However, until now, only a few transcription factors operating downstream of Pax6 responsible for generation of individual retinal cell types have been identified. In this study, we identified two transcription factors of the Onecut family, Onecut1 and Onecut2, as Pax6 downstream-acting factors. Onecut1 and Onecut2 were previously shown to be expressed in developing horizontal cells, retinal ganglion cells and cone photoreceptors; however, their role in differentiation of these cell types is poorly understood. In this study, we show that the horizontal cell genesis is severely disturbed in *Onecut*-deficient retinae. In single *Onecut1* and *Onecut2* mutants, the number of horizontal cells is dramatically reduced while horizontal cells are completely missing in the *Onecut1*/*Onecut2* compound mutant. Analysis of genes involved in the horizontal cell genesis such as Foxn4, Ptf1a, Prox1 and Lim1 showed that although horizontal cells are initially formed, they are not maintained in *Onecut*-deficient retinae. Taken together, this study suggests the model in which Pax6 regulates the maintenance of horizontal cells through the activation of Onecut1 and Onecut2 transcription factors.

Introduction

Proper visual perception is strictly dependent on coordinated differentiation and correct assembly of multiple cell types in the structure of the neuroretina. During the course of retinogenesis seven retinal cell types are generated from the common retinal progenitor cell (RPC) population (Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990) and further organized in three retinal layers: retinal ganglion cells (RGCs) in the ganglion cell layer (GCL); amacrine cells (ACs), bipolar cells, Müller glia cells and horizontal cells (HCs) in the inner nuclear layer (INL); rod and cone photoreceptors in the outer nuclear layer (ONL). The birth order is conserved among vertebrate species, suggesting the existence of a common molecular mechanism governing this process. It is largely accepted that as the retinogenesis proceeds, RPCs are exposed to the changing environment of extrinsic cues (Cepko, 1999) that in cooperation with intrinsic factors direct retinal neurogenesis (Brown et al., 1998; Cepko, 1999; Hatakeyama and Kageyama, 2004; Inoue et al., 2002; Lillien, 1995; Perron and Harris, 2000; Tomita et al., 1996). Among intrinsic factors, mainly transcription factors of the basic helix-loop-helix (bHLH) and homeodomain class have been found to promote a strong cell-autonomous bias toward particular cell fates whereas inhibiting others (Hatakeyama and Kageyama, 2004). Although genetic studies have identified many transcription factors involved in the process of retinogenesis, the complete map of gene regulatory networks (GRNs) that mediate differentiation of all individual cell types is not completely understood.

The paired and homeodomain transcription factor Pax6 plays a pivotal role in retinal development. It is expressed from very early stages, in all mitotically active RPCs (Walther and Gruss, 1991). Later, its expression is restricted to differentiated retinal ganglion cells, amacrine cells, horizontal cells and Müller glial cells (de Melo et al., 2003; Roesch et al., 2008). Pax6 is assumed to be required for RPC multipotency as its early inactivation results in failure of acquisition of any specific retinal cell fate (Klimova and Kozmik, 2014). Since Pax6 was found to control expression of several bHLH transcription factors, it has been suggested that Pax6 regulates the complex GRN in the retina (Hatakeyama and Kageyama, 2004; Klimova and Kozmik, 2014; Marquardt et al., 2001; Oron-Karni et al., 2008; Riesenberg et al., 2009). However, until now only a few Pax6 downstream-acting factors potentially accounting for the severe differentiation defect observed in *Pax6*-deficient retinae have been identified.

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Onecut proteins (Oc) belong to an ancient superclass of homeobox transcription factors. They are represented by three members in mammals, Onecut1 (Oc1), Onecut2 (Oc2) and Onecut (Oc3) (Jacquemin et al., 1999; Lannoy et al., 1998; Vanhorenbeeck et al., 2002). Originally, they were identified as factors controlling cell differentiation in endoderm-derived tissues such as liver and pancreas (Clotman et al., 2005, 2002; Jacquemin et al., 2000, 2003; Margagliotti et al., 2007; Pierreux et al., 2006). However, in the last decade, there has been growing evidence that Oc play a role in the nervous system development as well. Oc have been found to regulate neuronal identity, migration, organization and maintenance in different regions of the central nervous system (Audouard et al., 2013; Espana and Clotman, 2012a, b; Hodge et al., 2007; Chakrabarty et al., 2012; Roy et al., 2012; Stam et al., 2012). Among the neuronal tissues, their expression was also observed in the retina (Haworth and Latinkic, 2009; Wu et al., 2012). Although detailed analysis of Oc expression in the mouse revealed expression of Oc1 and Oc2 in developing retinal ganglion cells, horizontal cells and cone photoreceptors (Emerson et al., 2013; Wu et al., 2013, 2012) their role in differentiation of these retinal cell types has not been completely understood.

Horizontal cells represent a population of retinal interneurons that modulate signaling between photoreceptors and bipolar cells. Like other retinal cell types, HCs are derived from the RPC population (Turner et al., 1990). Transcription factors Foxn4, RORβ1, Ptf1a, Prox1, Ap2, Sall3 and Lim1 were found to be required for differentiation and proper localization of HCs in the retina (Bassett et al., 2012; de Melo et al., 2011; Dyer et al., 2003; Fujitani et al., 2006; Li et al., 2004; Liu et al., 2013; Nakhai et al., 2007; Poche et al., 2007). Foxn4, one of the earliest HC determinants, controls expression of Ptf1a and Prox1 to promote the HC fate (Fujitani et al., 2006; Li et al., 2004). However, it has been suggested that co-expression of Foxn4 and Pax6 in mitotic RPCs is crucial for acquisition of competence for the genesis of HCs (Li et al., 2004). Although the Foxn4 role in HC development has been well established (Fujitani et al., 2006; Li et al., 2004), a potential role of Pax6 remains largely elusive. Here we show that Pax6 regulates the HC development through activation of two transcription factors of the Oc transcription factor family, Oc1 and Oc2.

Materials and methods

Mouse lines

For retina-specific inactivation of *Pax6*, *α-Cre* (Marquardt et al., 2001), *mRx-Cre* (Klimova et al., 2013) and *Pax6*$^{fl/fl}$ (Klimova and Kozmik, 2014) mice were used. To inactivate *Oc1* and *Oc2*, *Oc1*$^{fl/fl}$ (Zhang et al., 2009), *Oc2*$^{+/-}$ (Clotman et al., 2005) mice were used.

Tissue collections and histology

Mouse embryos were harvested from timed pregnant females. The morning of vaginal plug was considered as embryonic day 0.5 (E0.5). Embryos were fixed in 2% or 4% paraformaldehyde (w/v) in PBS on ice for the time depending on embryonic stage (from 20 min to 4 h). Embryos were washed with cold PBS, cryopreserved by overnight incubation in 30% sucrose (w/w), frozen in OCT (Tissue Tek, Sekura Finetek) and sectioned.

Immunohistochemistry and β-galactosidase staining

The cryosections were permeabilized with PBT (PBS with 0.1% Tween-20) for 15 min, blocked with 10% BSA in PBT for 30 min and incubated with primary antibody overnight at 4 °C. Primary antibodies used were: rabbit anti-Pax6 (Covance, PRB-278P, 1:500), mouse anti-Pax6 (DSHB, clone P3U1, 1:2000), mouse anti-Pax6 (Santa-Cruz, sc-53108, 1:50), mouse anti-Prox1 (Millipore, MAB5652, 1:1000), mouse anti-Lim1/2 (DSHB, clone 4F2-c, 1:250), rabbit anti-Oc1 (HNF6) (Santa-Cruz, H-100, sc-13050, 1:500), sheep anti-Oc2 (R&D Systems, AF6294, 1:500), rabbit anti-Ptf1a (AB2153, provided by Dr. Ole Madsen, Beta Cell Biology Consortium, 1:250), goat anti-Brn3b (Santa-Cruz, C-13, sc-6026X, 1:2000), mouse anti-Isl1/2 (DSHB, clone 40.2D6-c, 1:200), rabbit anti-Brn3a (provided by Dr. E. Turner, 1:4000), mouse anti-Calbindin-D-28K (Sigma-Aldrich, clone CB-955, C9848, 1:2500), rabbit anti-Calretinin (Sigma-Aldrich, C7479, 1:750), rabbit anti-Ap2α (Santa-Cruz, C-18, sc-184, 1:1000), mouse anti-Ap2α (DSHB, clone 3B5, 1:1000), RXRγ (Santa-Cruz, Y-20, sc-555X, 1:3000), rabbit anti Tbr2 (Abcam, ab23345, 1:500). Sections were washed 3 × 10 min with PBT, incubated with secondary antibody (Molecular Probes) for 1 h, washed 3 × 20 min in PBT, washed 10 min with DAPI (1 μg/ml) in PBT and mounted into Mowiol (Sigma). The paraffin sections were deparaffinized and rehydrated. For immunohistochemical analysis, dewaxed sections were incubated for 20 min in citrate buffer (10 mM, pH 6.0) at 98 °C in a steam bath. Sections were washed 3 × 10 min with PBT, treated with 1.5% H_2O_2 in 10% methanol in PBS for 25 min, again washed 3 × 10 min with PBT, blocked with 10% BSA in PBT for one hour and incubated with primary antibody overnight at 4 °C. Primary antibody was detected with biotinylated anti-mouse, anti-rabbit secondary antibody (Vector Laboratories) and subsequently visualized with Vectastain ABC Elite kit and ImmPACT DAB substrate (all Vector Laboratories).

For β-galactosidase staining, embryos were fixed in 2% PFA, sections were washed with the rinse buffer (0.1 M phosphate buffer pH 7.3, 2 mM $MgCl_2$, 20 mM Tris pH 7.3, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40) and incubated in X-Gal staining solution (rinse buffer supplemented with 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 20 mM Tris pH 7.3, and 1 mg/ml X-gal) at 37 °C.

In situ hybridization

For RNA antisense probe synthesis, plasmid carrying Foxn4 cDNA (nucleotides 492–1562 of NM_148935) was used. The antisense mRNA probe was synthesized using RNA polymerase and digoxigenin-labeled nucleotides (Roche) according to the manufacturer's instructions. RNA *in situ* hybridization was carried out as previously described (Fujimura et al., 2009).

Quantitative RT-PCR (qRT-PCR)

For analysis of differentially expressed genes in wild-type and Pax6-deficient retinal cells, E12.5 embryonic eyes were dissected and washed in cold PBS and treated with 0.5% trypsin in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Life Technologies) for 4 min at 37 °C to prepare single-cell suspension. Cells were subsequently washed with DMEM supplemented with 20% fetal bovine serum (FBS) (Gibco, Life Technologies), transferred to FBS-free DMEM, and EGFP$^+$ retinal cells were FACS sorted. FACS-sorted cells were subjected to RNA isolation using the RNeasy Micro isolation system (Qiagen) according to the standard manufacturer's protocol. Isolated RNA was subjected to reverse transcription and amplification using Ovation® Pico WTA Systems V2 (Nugene) and the resulting cDNA was used for qRT-PCR. Two independent samples for both wild-type and Pax6-deficient retinae were used. Every sample was represented by 20 embryonic eyes originating from 2–3 litters collected in one experiment.

To analyze mRNA expression in Onecut mutant retinae, retinae from E14.5 embryonic eyes were dissected and total RNA was isolated with Trizol® Reagent (Life Technologies). Random-primed cDNA was generated from 200 ng of total RNA using SuperScript VILO cDNA Synthesis kit (Life Technologies). At least four different

embryos originating from two litters were used for retina dissection and subsequent RNA isolation per each genotype.

qRT-PCR was run in the LightCycler® 480 Instrument (Roche) using a LightCycler® 480 DNA SYBR Green I Master (Roche) according to the standard manufacturer's protocol; typically, 5 μl reaction mixture was used. PCR reactions were performed in technical duplicate for each primer set of primers, with four different cDNAs. Crossing point (Cp) values were calculated by LightCycler® 480 Software (Roche) using the second-derivate maximum algorithm. The average Cp values of all technical replicates were normalized by Cp values of a housekeeping gene. Statistical significance of the change in mRNA expression was calculated by a two-tailed Student's *t*-test. Finally, the change in mRNA expression was presented as the ratio $Oc1^{-/-}$/wild-type ($Oc2^{-/-}$/wild-type, $Oc1^{-/-}/Oc2^{-/-}$/wild-type) retinae in log with base 2 scale. Sequences of used primers are listed in Supplementary Table 1.

Quantification of marker-positive cells

To analyze the number of HCs, eyes were sectioned, immunostained, and the number of marker-positive HCs per whole central retinal section was counted and normalized to wild-type control. For a single eye, a minimum of five sections were used; for each genotype, a minimum of eight individual retinae was analyzed. Statistical significance was assessed by Student's *t*-test.

To analyze the number of other retinal cell types, the number of marker-positive cells from a single eye field of the central retinal section was counted. Eye fields localized in the same distance from the optic nerve were selected. The number of marker-positive cells per defined retinal area was counted and normalized to wild-type control. For a single eye, a minimum of five eye fields; for each genotype, a minimum of four retinae were used. Statistical significance was analyzed by Student's *t*-test.

Electrophoretic mobility shift assays (EMSAs)

In silico analysis to identify putative Pax6 binding sites in *Oc1* locus was performed using high-quality transcription factor binding profile database JASPAR (http://jaspar.genereg.net; JASPAR: an open-access database for eukaryotic transcription factor binding profiles) (Sandelin et al., 2004). Electrophoretic mobility shift assays (EMSAs) with the full-length Pax6 was performed as previously described (Kozmik et al., 1997) using double-stranded oligonucleotides comprising binding sites shown in Supplementary Table 1. For competition experiment, wild-type and mutated Pax6 consensus binding sites were used as specific or non-specific competitor, respectively.

Cell culture, transient transfection and luciferase reporter assay

293T cells were cultured in Dulbecco's modified Eagle's medium (SIGMA) supplemented with 10% fetal bovine serum (PAA laboratories), 2 mM L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin (SIGMA). Transient transfection was performed using Fugene 6 (Roche) according to the manufacturer's protocol. Cells were plated in 24-well plates 24 h prior to transfection. Typically, 100 ng of the firefly luciferase reporter gene containing sites BC and DE introduced upstream of minimal TATA containing promoter in pGL3 vector (Kozmik et al., 1997) was co-transfected with 50 ng of Pax6 expression plasmid. The total amount of DNA transfected per well was 300 ng and was adjusted with pUC18. A β-galactosidase expression plasmid was co-transfected to normalize the transfection efficiency. Triplicate assays were performed to obtain standard deviations. Two days after transfection, the cells were lysed in 100 μl of 1 × passive lysis buffer (Promega). Luciferase reporter assays were performed using a Luciferase Reporter assay kit (Promega). β-galactosidase was detected with the Galacto-Star system (Applied Biosystems).

Results

Oc1 and Oc2 transcription factors operate downstream of Pax6 in retinal development

Pax6 plays a pivotal role in retinal development as *Pax6*-deficient retinae display severe differentiation defects (Klimova and Kozmik, 2014; Marquardt et al., 2001). This is by large attributed to the Pax6 ability to regulate expression of the transcription factors that mediate differentiation of particular retinal cell types (Marquardt et al., 2001; Oron-Karni et al., 2008; Riesenberg et al., 2009). Although some Pax6 downstream-acting genes have been previously identified, the complete transcriptional network leading from Pax6-expressing RPCs to all differentiated retinal cell types remains unresolved. In the search for Pax6-regulated genes in mouse retinal development, we screened Pax6 wild-type and Pax6-deficient retinal cells for differentially expressed genes (Fig. 1A). To inactivate *Pax6* in retinal cells, we generated conditional *Pax6* retinal mutants. We used mice with floxed *Pax6* allele ($Pax6^{flox/flox}$) (Klimova and Kozmik, 2014) and distal retina-specific *α-Cre* mice as a deleter (Marquardt et al., 2001) to generate $Pax6^{flox/flox}$/*α-Cre* ($Pax6^{-/-}$) retinal mutants. The *α-Cre*-mediated recombination leads to Pax6 elimination in distal parts of the retina at E12.5. At this stage the subpopulation of RPCs proceeds through the differentiation process, and thus analysis of this stage enables identification of both progenitor-cell-specific and differentiation-specific genes. As EGFP is expressed along with the Cre recombinase from *α-Cre* transgene (Marquardt et al., 2001), $EGFP^{+}/Pax6^{-/-}$ retinal cells were FACS sorted from E12.5 $Pax6^{flox/flox}$/*α-Cre* embryonic eyes and subjected to qRT-PCR for candidate Pax6-regulated genes (Fig. 1A). As a control, Pax6 wild-type retinal cells from $Pax6^{wt/wt}$/*α-Cre* ($EGFP^{+}/Pax6^{+/+}$) eyes were used. We established several criteria for Pax6-regulated candidate genes. We searched for: i) evolutionarily conserved ii) neuronal tissue-specific iii) transcriptional factors and iv) with a role in cell type specification. Based on these criteria, Oc1 and Oc2 transcription factors were identified as potential Pax6-regulated genes. The qRT-PCR performed with wild-type and Pax6-deficient retinal cells at E12.5 clearly showed a decrease of *Oc1* and *Oc2* mRNA expression corresponding with decreased expression of *Pax6* (Fig. 1B). Accordingly, immunohistochemical analysis showed a dramatic decrease of Oc1 and Oc2 protein levels (Fig. 1C). Although the expression of both Oc1 and Oc2 proteins was apparent in Pax6 wild-type ($Pax6^{flox/flox}$) retinae at E13.5, their expression was lost in the distal parts of Pax6 mutant ($Pax6^{flox/flox}$/α-*Cre*) retinae (Fig. 1C).

Oc1 and Oc2 proteins were previously found to be co-expressed in developing retinal ganglion cells and horizontal cells (Wu et al., 2012). The main prerequisite for target gene regulation is co-expression of selected proteins within the identical cell population. To find out whether Pax6 and Oc1 and Oc2 proteins are co-expressed during retinal development, retinal sections from E14.5 and postnatal (P18) retinae were co-stained with Pax6 and Oc1 or Pax6 and Oc2 antibody (Fig. 2). At E14.5 Pax6 is expressed virtually by all retinal cells and its co-expression with Oc1 and Oc2 was apparent in all Oc1 and Oc2-expressing cells (Fig. 2A and B). Based on previous observations, these cells represent cycling RPCs or differentiating ganglion and horizontal cells (Wu et al., 2012). In postnatal retinae (P18), Pax6 was expressed in RGCs localized in GCL and ACs and HCs localized in INL (Fig. 2C and D). At the same stage, Oc2 along with Oc1 was expressed in the HC population, where they were co-expressed with Pax6 (Fig. 2C–E). These data show that transcription factors Oc1 and Oc2 are co-expressed with Pax6 within the same populations during retinal development. Moreover, the absence of *Oc1* and *Oc2* gene products in Pax6-deficient retinal cells strongly indicates that *Oc1* and *Oc2* operate downstream of Pax6.

To provide a more direct link between Pax6 and Onecut expression, we performed *in silico* analysis of the upstream

regulatory region of *Oc1* and revealed several putative binding sites for Pax6 (Fig. 3A and B). Binding of the full-length Pax6 to individual sites was experimentally verified by electrophoretic mobility shift assays (EMSAs) in the presence of specific and nonspecific competitor DNA (Fig. 3C). Putative binding sites located in the −8 kb (site A), −4 kb (sites B and C) and −2.5 kb (sites D and E) region upstream of the *Oc1* gene transcription start site were recognized by full-length Pax6 specifically, since complex formation was efficiently inhibited by the presence of unlabeled Pax6 consensus binding site but not in the presence of mutant Pax6 binding site (Fig. 3C). To see whether the sites identified in the upstream regulatory region of *Oc1* gene by EMSA can function as Pax6-dependent regulatory modules, their sequences (listed in Supplementary Table 1) were introduced into the minimal reporter gene containing TATA element and such reporters were tested by co-transfection with an expression vector encoding Pax6. As shown in Fig. 3D, Pax6 activates transcription of reporter genes carrying sites BC and DE but the reporter gene is devoid of any binding site. Combined, our data show that the upstream regulatory region of *Oc1* gene contains several binding sites that are recognized by Pax6 *in vitro* and that can mediate Pax6-dependent regulation in transient reporter assays *in vivo*.

Oc1 and Oc2 are essential for horizontal cell development

Oc1 and Oc2 have been previously found to be expressed during the development of RGCs, HCs and cone photoreceptors in mammalian retina (Emerson et al., 2013; Wu et al., 2012). To investigate their potential role in differentiation of these retinal cell types, *Oc1* and *Oc2* were inactivated in the mouse retina (Fig. 4). To inactivate *Oc1*, mice with the floxed allele of *Oc1* ($Oc1^{flox/flox}$) (Zhang et al., 2009) were crossed with retina-specific *mRx-Cre* (Klimova et al., 2013) to generate $Oc1^{flox/flox}$/*mRx-Cre* ($Oc1^{-/-}$) retinal mutants. In *mRx-Cre* the Cre-mediated recombination is performed in the presumptive retinal tissue at E9.5. This enables inactivation of *Oc1* early before the differentiation program is initiated (Klimova et al., 2013). To inactivate *Oc2*, $Oc2^{+/-}$ mice (Clotman et al., 2005) were used. Since $Oc2^{-/-}$ mice fail to thrive during early postnatal period and display high levels of mortality before weaning (Dusing et al., 2010), heterozygote knockout mice were crossbred to obtain $Oc2^{-/-}$ embryos and postnatal mice. To inactivate *Oc1* along with *Oc2* to generate $Oc1^{-/-}/Oc2^{-/-}$ retinae, $Oc2^{+/-}$ mice were crossed to $Oc1^{flox/flox}$/*mRx-Cre* background. The efficiency of Oc1 and Oc2 inactivation was analyzed by immunohistochemistry of E14.5 retinal sections using Oc1 and Oc2-specific antibody (Fig. 4A–H). Although Oc1 and Oc2 expression was apparent in wild-type retinae (Fig. 4A and E), their expression was lost in corresponding mutants (Fig. 4B–D, F–H). The consequence of Oc1 and Oc2 inactivation was first investigated at the histological level in retinae from $Oc1^{-/-}$, $Oc2^{-/-}$ and $Oc1^{-/-}/Oc2^{-/-}$ mice at P18, at the stage when retinogenesis is complete. Although the wild-type retina displayed proper lamination with distinctly formed layers of retinal cells (Fig. 4I), in $Oc1^{-/-}$ and $Oc2^{-/-}$ retinae, the outer plexiform layer (opl), localized between INL and ONL, was thinned (Fig. 4J and K). In $Oc1^{-/-}/Oc2^{-/-}$ double mutant the opl was not even distinguishable and INL and ONL fused to form a single layer (Fig. 4L). In additionally, INL and inner plexiform layer (ipl) of the $Oc1^{-/-}/Oc2^{-/-}$ double

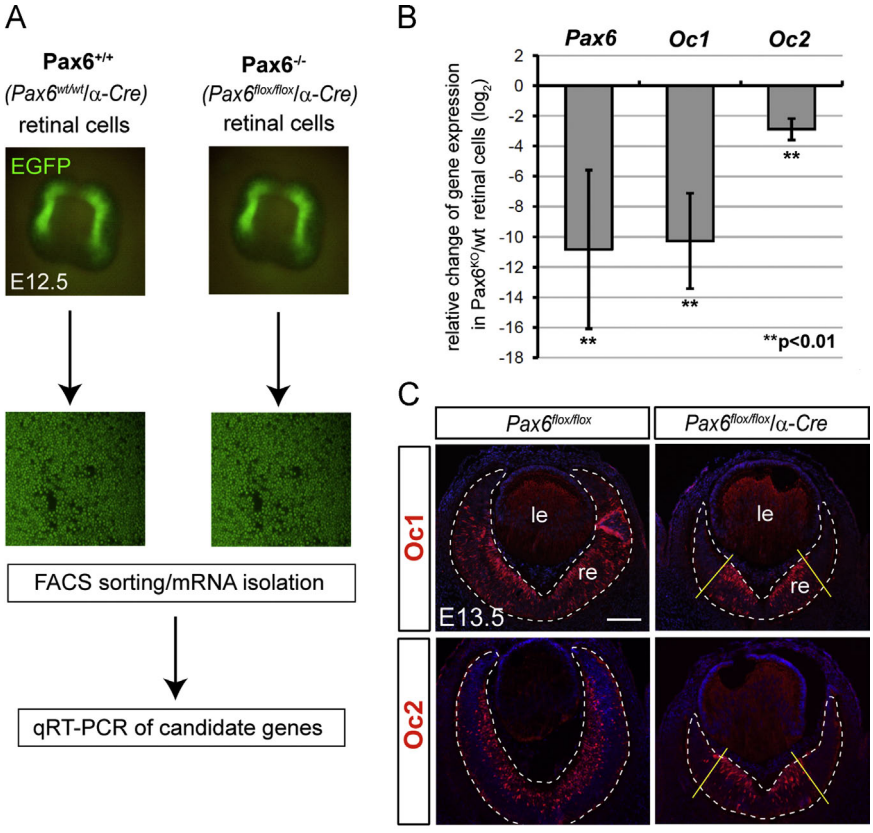


Fig. 1. Transcription factors Oc1 and Oc2 operate downstream of Pax6 in retinal development. (A) Experimental strategy used to search for Pax6-regulated genes in retinal development. E12.5 embryonic eyes of $Pax6^{wt/wt}$/*α-Cre* Pax6 wild-type ($EGFP^{+}/Pax6^{+/+}$) and $Pax6^{flox/flox}$/*α-Cre* Pax6 retinal mutants ($EGFP^{+}/Pax6^{-/-}$) were dissected, transformed to single-cell suspension and subjected to FACS to obtain $Pax6^{+/+}$ and $Pax6^{-/-}$ retinal cells. mRNA from $Pax6^{+/+}$ and $Pax6^{-/-}$ retinal cells were isolated and processed by qRT-PCR using primers specific for candidate Pax6-regulated genes. (B) qRT-PCR analysis showing the changes of relative mRNA expression of *Pax6* and candidate transcription factors *Oc1* and *Oc2* in $Pax6^{+/+}$ and $Pax6^{-/-}$ (Pax6KO) retinal cells. Error bars indicate standard deviation, *p*-Values are calculated by Student's *t*-test. (C) Immunohistochemical detection of Oc1 and Oc2 proteins in Pax6 wild-type ($Pax6^{flox/flox}$) and Pax6-deficient ($Pax6^{flox/flox}$/*α-Cre*) distal retina. The area of α-Cre-mediated inactivation of *Pax6* in $Pax6^{flox/flox}$/*α-Cre* retinae is demarcated with a solid yellow line. Retina is indicated with a dashed line. le, lens; re, retina.

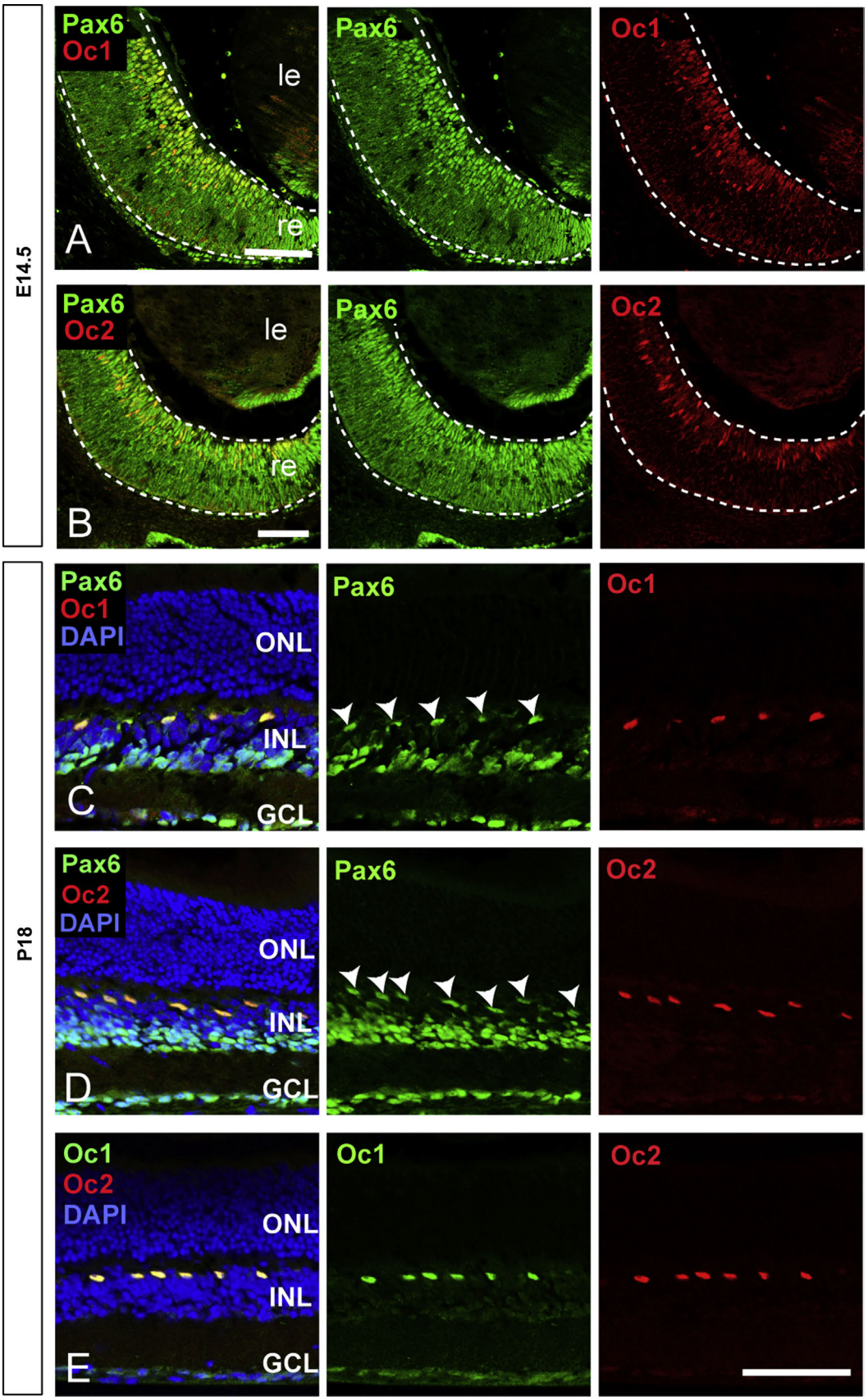


Fig. 2. Oc1 and Oc2 are co-expressed with Pax6 during retinal development. (A, B) Retinal sections of E14.5 eyes stained with Pax6-, Oc1- and Oc2-specific antibody showing the co-expression of Pax6 and Oc1 (A) and Pax6 and Oc2 (B) in retinal cells. (C–E) Retinal sections of P18 eyes stained with Pax6-, Oc1- and Oc2-specific antibody showing co-expression of Pax6 and Oc1 (C), Pax6 and Oc2 (D) and Oc1 and Oc2 (E) in horizontal cells localized between the inner and outer nuclear layers. Retinae at E14.5 are indicated with a dashed line. Arrowheads in (C) and (D) indicate cells that co-express Pax6 and Oc1 or Oc2, respectively. le, lens; re, retina; RPE, retinal pigmented epithelium; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

mutant was thinned as well (Fig. 4L). As the opl is the layer where the projections of horizontal cells are normally present and HCs are the only Oc-expressing cell type in the adult retina (Wu et al., 2012), histological analysis indicated a defect in the HC population. To analyze whether HCs are present in mutant retinae, sections of *Oc1*$^{-/-}$, *Oc2*$^{-/-}$, *Oc1*$^{-/-}$/*Oc2*$^{-/-}$ and wild-type P18 eyes were analyzed for expression of HC-specific markers (Fig. 5). A calcium binding protein, Calbindin, is expressed in all HCs and a subset of amacrine cells (Dyer et al., 2003; Poche et al., 2007), along with activating protein-2 Ap2α (Bassett et al., 2012, 2007). On the other hand, expression of lim-homeodomain transcription factor Lim1, Oc1 and Oc2 is found exclusively in HCs at this stage (Liu et al., 2000; Poche et al., 2007; Wu et al., 2012). Staining for all indicated proteins was dramatically reduced in all mutants when compared with the wild-type control (Fig. 5A) indicating a defect in HC development. To quantify the decrease of horizontal cells in particular mutants, retinal sections were stained with Ap2α antibody and the number of Ap2α^{+} cells localized at the outer face of the INL was counted. Whereas the quantification showed a dramatic decrease of HCs in single *Oc1*$^{-/-}$ and *Oc2*$^{-/-}$ mutants (80.2 ± 2.8% for *Oc1*$^{-/-}$; 84.7 ± 4.3% for *Oc2*$^{-/-}$), in retinae of double mutants, only a few if any Ap2α^{+} HCs were found (Fig. 5B). The same result was obtained when Calbindin was used for quantification of HCs (data not shown). These data show that Oc1 and Oc2 inactivation leads to HC loss.

Previous study has shown that apart from HCs, Oc1 and Oc2 are strongly expressed by differentiating RGCs (Wu et al., 2012). It has been suggested that Oc proteins might play an important role during RGC differentiation as well (Wu et al., 2012). Based on that, we selected several markers of RGCs and assessed *Oc1*$^{-/-}$, *Oc2*$^{-/-}$, *Oc1*$^{-/-}$/*Oc2*$^{-/-}$ and wild-type E14.5 and P18 retinae for their expression (Supplementary Fig. S1). At E14.5, immunohistochemistry was used to analyze the protein expression of Brn3a, Brn3b, Isl1 and Tbr2 (Supplementary Fig. S1A) and qRT-PCR was used to assess the expression of *Pax6*, *Math5*, *Brn3b*, *Isl1*, *Tbr2*, *Oc1*, *Oc2* (Supplementary Fig. S1B). At P18, immunohistochemistry was used to analyze the expression of Brn3a, Brn3b, Isl1, Tbr2 and Pax6 (Supplementary Fig. S1C) and the number of Brn3b^{+}, Pax6^{+} and Tbr2^{+} cells localized in GCL was counted to quantify the number of RGCs (Supplementary Fig. S1D). This analysis showed that mRNA expression of *Oc1* and *Oc2* was dramatically reduced in the corresponding mutants (Supplementary Fig. S1B), as expected, and their expression levels in particular mutants did not indicate any interaction between *Oc1* and *Oc2*. Along with *Oc1* and *Oc2*, the expression of the other Onecut factor, *Oc3* was dramatically reduced in the *Oc1*$^{-/-}$/*Oc2*$^{-/-}$ double mutant (Supplementary Fig. S1B), indicating *Oc3* dependence on *Oc1* and *Oc2* expression. Although immunohistochemical analysis at E14.5 and P18 and qRT-PCR at E14.5 did not reveal any conspicuous difference in the expression of *Math5*, *Brn3a*, *Brn3b* and *Isl1* (Supplementary Fig. S1A–C), the expression of *Tbr2* was dramatically reduced in Oc-deficient retinae (Supplementary Fig. S1A–D). At E14.5, Tbr2 was not detectable and the number of Tbr2^{+} cells at P18 was reduced by 63% (± 10.9%) in the *Oc1*$^{-/-}$/*Oc2*$^{-/-}$ double mutant retinae

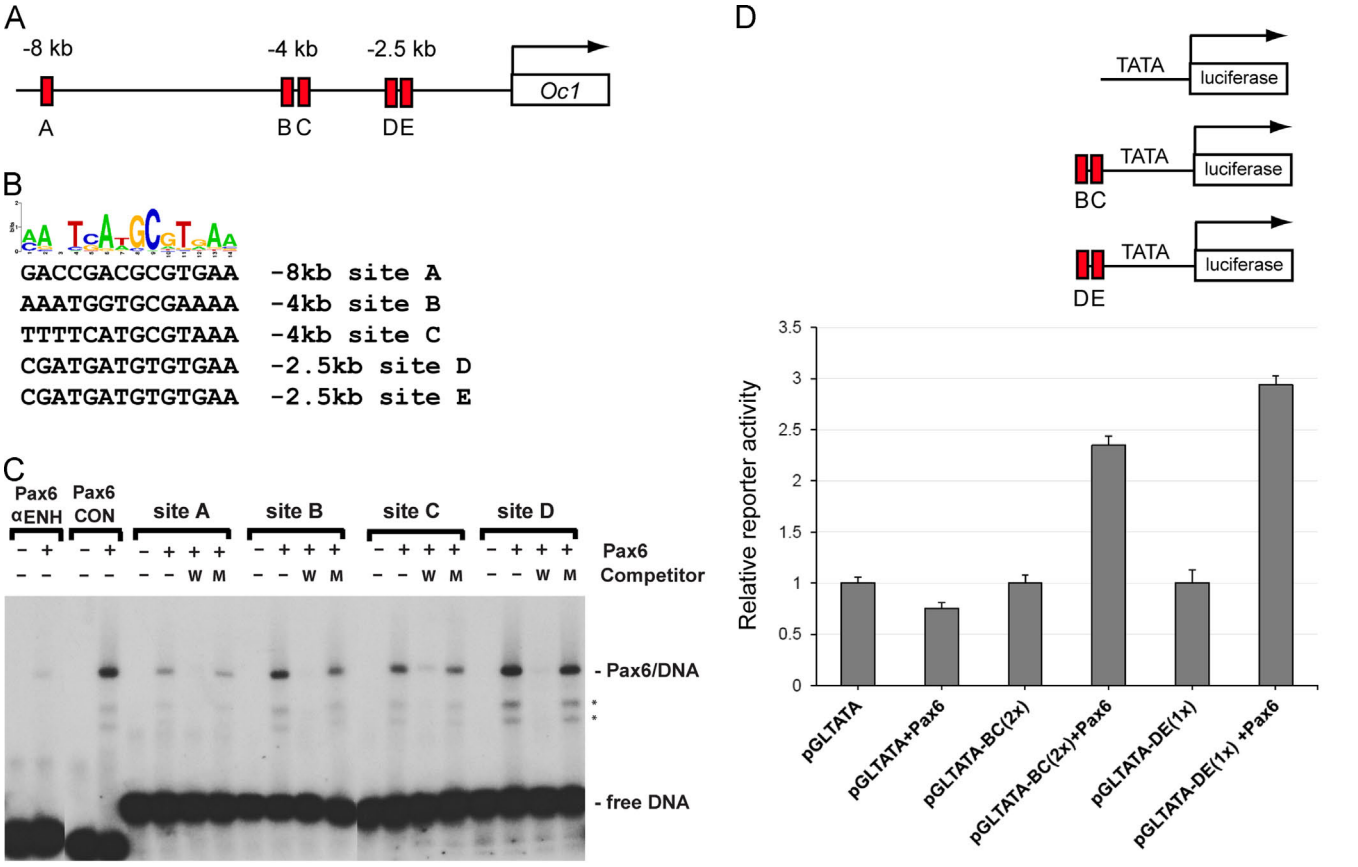


Fig. 3. Multiple Pax6 binding sites are present in the upstream regulatory region of *Oc1* gene. (A) Schematic diagram of *Oc1* upstream regulatory region indicating position of Pax6 binding sites A–E. (B) Alignment of sites A–E with the recognition sequence logo of Pax6. (C) Electrophoretic mobility shift assay (EMSA) with binding sites A–D (nucleotide sequence of Pax6 binding site E is identical to site D). The specific complex of Pax6 with its binding sites is competed by wild-type Pax6 consensus site (W) but not by the mutant binding site (M). Asterisks denote DNA-binding competent degradation products of Pax6. Binding to a functional Pax6 binding site derived from retina-specific α enhancer (Schwarz et al., 2000) and high-affinity consensus Pax6 binding site is shown for comparison. (D) Indicated luciferase reporter genes were co-transfected with Pax6-encoding expression vector or with empty expression vector. Triplicate assays were performed to obtain standard deviations and transfection efficiency was normalized by co-transfection of β-galactosidase expression plasmid.

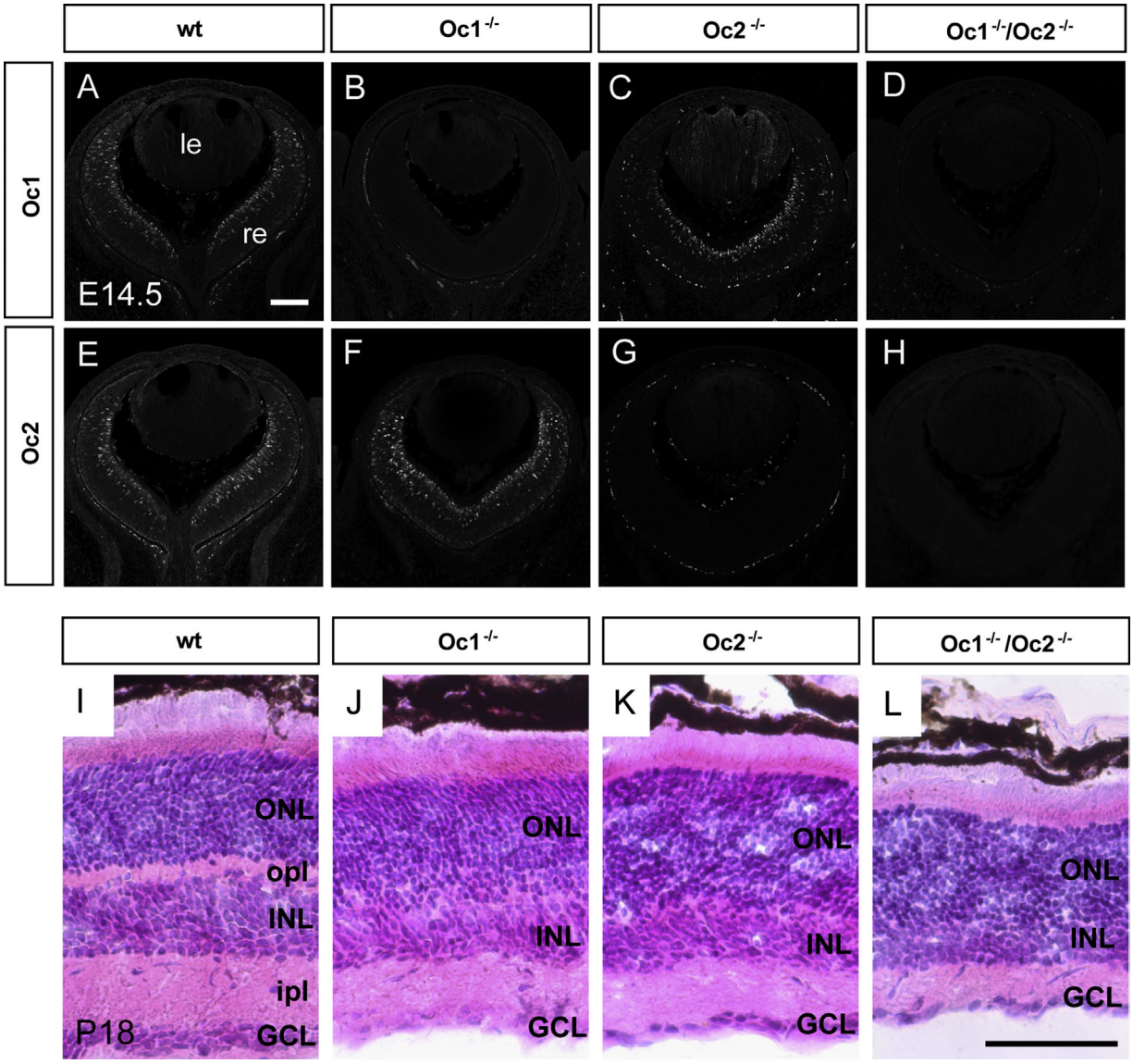


Fig. 4. Oc1 and Oc2 inactivation in the mouse retina. (A–H) Retinal sections of E14.5 wild-type (*wt*), $Oc1^{-/-}$, $Oc2^{-/-}$ and $Oc1^{-/-}/Oc2^{-/-}$ eye stained with Oc1- and Oc2-specific antibody showing the efficiency of Oc protein elimination in the corresponding mutants. (I–L) Retinal sections of P18 wild-type (*wt*), $Oc1^{-/-}$, $Oc2^{-/-}$ and $Oc1^{-/-}/Oc2^{-/-}$ eyes stained with hematoxylin-eosin showing a reduced outer plexiform layer in Oc mutants. le, lens; re, retina; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; opl; outer plexiform layer; ipl, inner plexiform layer.

(Supplementary Fig. S1A, C and D). However, quantification of RGCs at P18 showed no difference in the number of $Brn3a^+$ RGCs and only a slight reduction of $Pax6^+$ RGCs (by $9.8\% \pm 1.1\%$) (Supplementary Fig. S1D) in $Oc1^{-/-}/Oc2^{-/-}$ double mutant retina. Although Pax6 is expressed by most RGCs and Brn3a by ~80% of RGCs (Xiang et al., 1995), Tbr2 is expressed by a small subset of RGCs (~10%) (Mao et al., 2008; Sweeney et al., 2014). Our data indicate that Oc proteins are involved in generation of $Tbr2^+$ RGCs.

Recently, Oc factors have been found to be involved in cone differentiation in mouse retina (Emerson et al., 2013; Sapkota et al., 2014). Thus we examined the expression of an early cone-specific marker Rxrγ (Roberts et al., 2005) in E14.5 and P18 wild-type and Oc-deficient retinae. In accordance with previous works, we found Rxrγ protein expression absent in $Oc1^{-/-}/Oc2^{-/-}$ E14.5 (Supplemetary Fig. S2A) and quantification at P18 showed decreased number of $Rxr\gamma^+$ cells (by 23% $\pm 1.9\%$) in $Oc1^{-/-}/Oc2^{-/-}$ retinae indicating that number of cone photoreceptors is reduced upon Oc inactivation. Among other retinal cell types, we observed decreased numbers of ACs immunoreactive for Calretinin (decreased by $61.5 \pm 0.5\%$), Pax6 (decreased by $17.6 \pm 4.8\%$) and Isl1 (decreased by $25 \pm 1.6\%$). As recent study revealed defects in generation of other retinal cell types as well (Sapkota et al., 2014) we additionally analyzed the number of bipolar (BC) and Müller glia cells in Oc-deficient retinae. BCs immunoreactive for Chx10 were decreased by $27 \pm 3.7\%$ and Müller glia cells immunoreactive for Lhx2 were decreased by $27 \pm 2.7\%$ (Supplementary Fig. S2B) in $Oc1^{-/-}/Oc2^{-/-}$ retinae. Although for Calretinin, Pax6, Isl1 and Chx10, the decrease was observed in the $Oc1^{-/-}/Oc2^{-/-}$ double mutant only, for Lhx2, the decrease was observed in single Oc1 and Oc2-deficient retinae as well. As ACs, BCs and Müller glial cells are present in the INL, their reduction might contribute to the reduced thickness of INL and ipl observed in $Oc1^{-/-}/Oc2^{-/-}$ double mutant retinae (Fig. 4L). Taken together, our data indicate that Oc loss has an impact on majority of retinal cell types.

Although HCs are absent postnatally, their differentiation program is initiated in Oc-deficient embryonic retinae

Like other retinal cell types, HCs are generated from a common RPC population. Several transcription factors have been shown to

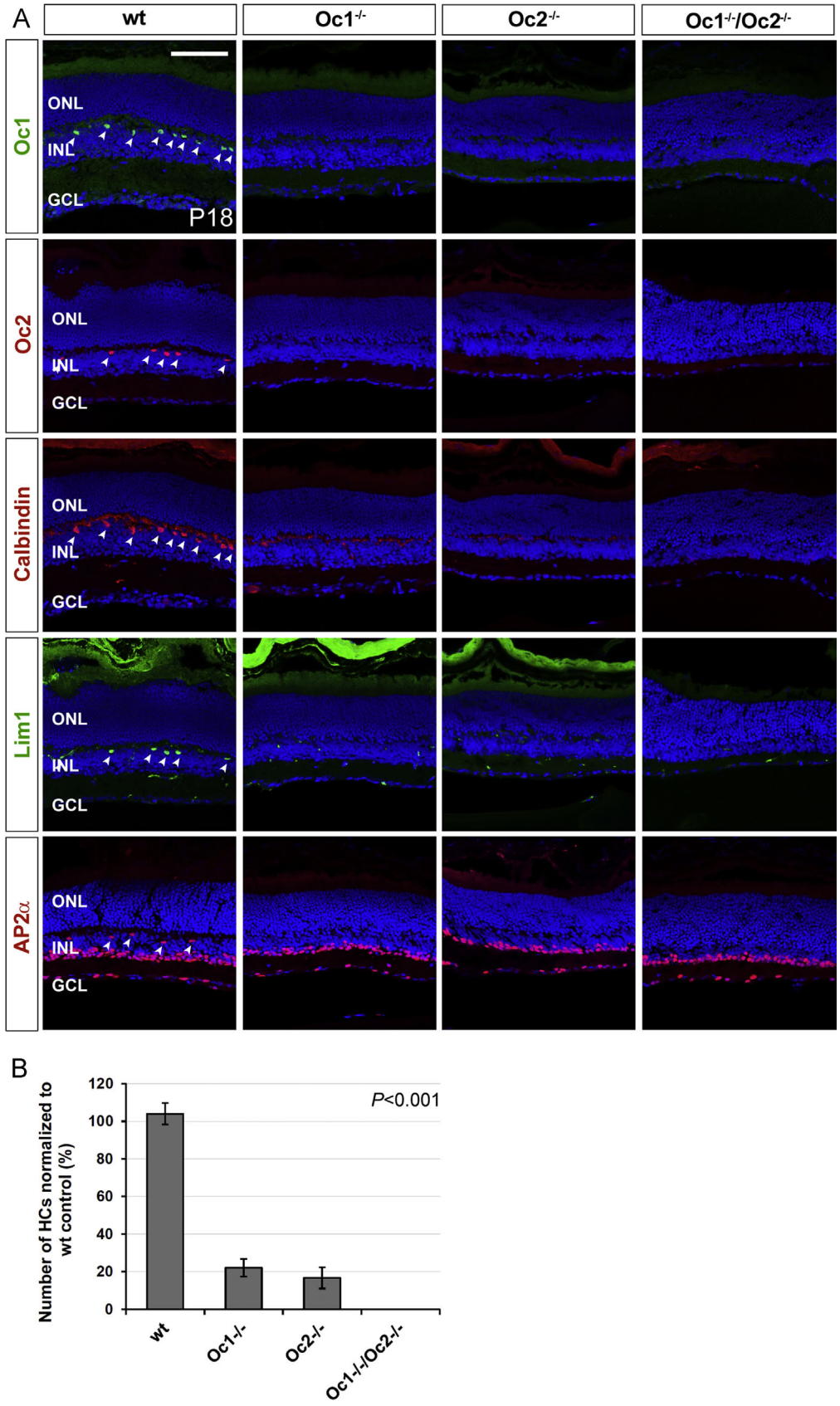


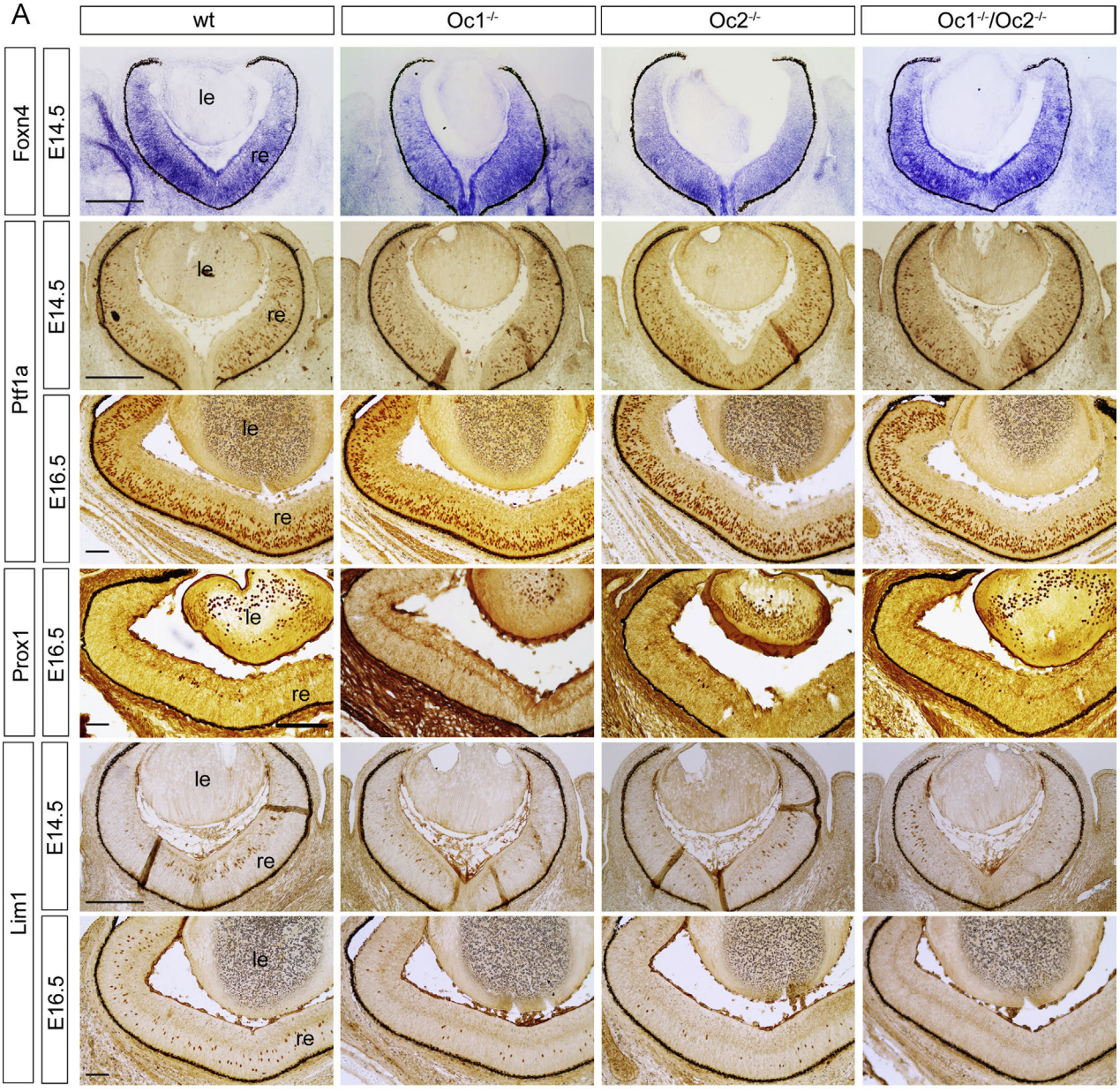
Fig. 5. Oc1 and Oc2 are essential for horizontal cell development. (A) Retinal section of P18 wild-type (*wt*), $Oc1^{-/-}$, $Oc2^{-/-}$ and $Oc1^{-/-}/Oc2^{-/-}$ eyes stained with antibodies against HC markers Oc1, Oc2, Calbindin, Lim1 and Ap2α. (B) Quantification of the number of HCs in wild-type (*wt*), $Oc1^{-/-}$, $Oc2^{-/-}$, $Oc1^{-/-}/Oc2^{-/-}$ and $Oc1^{+/-}/Oc2^{+/-}$ retinae assessed as the number of $Ap2\alpha^{+}$ cells localized at the outer face of the inner nuclear layer. Error bars indicate standard deviation, *p*-values are calculated by Student's *t*-test. Arrowheads indicate HCs stained for HC-specific marker. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

be required for HC development, including Foxn4, Ptf1a, Prox1 and Lim1 (Dyer et al., 2003; Fujitani et al., 2006; Li et al., 2004; Nakhai et al., 2007). HCs share initial steps of differentiation with ACs. In HC/AC common precursors, Foxn4 operates upstream of Ptf1a to control both HC and AC fates (Fujitani et al., 2006; Li et al., 2004). On the other hand, transcription factors Prox1 and Lim1 are required specifically for development and proper laminar positioning of HCs (Dyer et al., 2003; Poche et al., 2007). As simultaneous Oc1 and Oc2 inactivation is associated with complete loss of HCs, we analyzed the relationship of Oc1 and Oc2 with other transcription factors implicated in HC development. We assessed retinae from E14.5 and E16.5 wild-type, *Oc1*$^{-/-}$, *Oc2*$^{-/-}$ and *Oc1*$^{-/-}$/*Oc2*$^{-/-}$ mutant retinae for the protein expression of Ptf1a, Lim1 and Prox1 (Fig. 6A, Supplementary Fig. S3). *Foxn4* expression was analyzed by *in situ* hybridization at E14.5 (Fig. 6A). Finally, we processed mRNA from E14.5 wild-type, *Oc1*$^{-/-}$, *Oc2*$^{-/-}$ and *Oc1*$^{-/-}$/*Oc2*$^{-/-}$ mutant retinae by qRT-PCR to quantify the mRNA expression of *Foxn4*, *Ptf1a*, *Prox1* and *Lim1* (Fig. 6B). Embryonal stages for analysis of particular gene products were selected with respect to the onset of their expression and the onset of HC genesis. Whereas qRT-PCR and immunohistochemical analysis did not reveal any significant changes of *Foxn4* and *Ptf1a* expression, the expression of *Lim1* was reduced in *Oc1*$^{-/-}$, *Oc2*$^{-/-}$ and *Oc1*$^{-/-}$/*Oc2*$^{-/-}$ retinae (Fig. 6A and B). At the mRNA level, slight reduction of *Lim1* was apparent at E14.5 (Fig. 6B), whereas Lim1 protein expression remained unchanged at this stage when compared with wild-type control (Fig. 6A). At E16.5, the Lim1 protein expression was reduced in *Oc1*$^{-/-}$ and *Oc2*$^{-/-}$ with no expression observed in *Oc1*$^{-/-}$/*Oc2*$^{-/-}$ mutant retinae (Fig. 6A). These data indicate that although the Lim1 expression was properly initiated, it was not maintained in Oc-deficient retinae. For *Prox1*, qRT-PCR did not indicate any change in the mRNA expression (Fig. 6B). Immunohistochemical analysis performed at E14.5 and E16.5 showed that at both stages Prox1 protein expression was maintained, although slightly weaker expression was observed at E14.5 (Fig. 6A; Supplementary Fig. S3). As Prox1^{+}/Lim1^{+} HCs are initially generated in Oc-deficient retinae, the data presented above indicate that although Oc1 and Oc2 factors are essential for HC development, they do not regulate the HC development through activation of Foxn4, Ptf1a, Prox1 or Lim1. To introduce Oc1 and Oc2 factors to the existing GRN of HC development, we finally analyzed the relationship between Pax6 and Foxn4 as Oc1 has been previously found to be dependent on Foxn4 expression as well (Wu et al., 2013). The qRT-PCR performed with E12.5 FACS-sorted Pax6$^{-/-}$ (*Pax6*$^{flox/flox}$/*α-Cre*) and Pax6$^{+/+}$ (*Pax6*$^{wt/wt}$/*α-Cre*) retinal cells showed that the expression of *Foxn4* is not dependent on Pax6 (Supplementary Fig. S4), indicating a Foxn4-independent mechanism of Pax6-mediated Oc1 and Oc2 activations.

As Lim1 expression was lost soon after HCs have been generated in Oc-deficient retinae, Oc factors might regulate HC development through the maintenance of HC-specific expression at later stages. To examine when HCs are lost, we analyzed E18.5 wild-type and Oc-deficient retinae for the expression of HC-specific genes *Prox1* and *Oc2* (Fig. 7). As *Oc2* knockout mice were generated by the insertion of *LacZ* into the *Oc2* coding sequence (Clotman et al., 2005), we used β-galactosidase expression to trace the expression of *Oc2*. In E18.5 wild-type (*Oc2*$^{+/-}$) retinae, the expression of Prox1 and β-galactosidase was apparent in the outer neuroblastic layer where HCs are localized (Fig. 7). However, in *Oc1*$^{-/-}$ and *Oc2*$^{-/-}$ retinae, number of Prox1^{+} cells was reduced and in *Oc1*$^{-/-}$/*Oc2*$^{-/-}$ mutant retinae Prox1 expression was not detectable and HCs were lost as no β-galactosidase staining was observed in corresponding retinal area (Fig. 7). These data indicated that HCs were lost soon after they have been generated and that Oc1/Oc2 play the role in HC maintenance.

Discussion

The transcription factor Pax6 is essential for eye development in various animal species (Kozmik, 2005). Studies in which the conditional gene inactivation approach was used showed that in mammalian retina Pax6 plays a central role in RPC multipotency (Klimova and Kozmik, 2014; Marquardt et al., 2001; Oron-Karni et al., 2008). Although generation of all retinal cell types requires Pax6, little is known about the identity of Pax6 downstream-acting factors that mediate differentiation of the particular retinal cell types. Previous studies showed that several pro-neural bHLH transcription factors including Math5, Ngn2, Mash1, Math3 and Neurod1 are dependent on Pax6 expression in the mouse retina (Klimova and Kozmik, 2014; Marquardt et al., 2001; Riesenberg et al., 2009). Accordingly, the enhancer element of *Math5* was found to be bound by Pax6, suggesting direct regulation by Pax6 (Marquardt et al., 2001; Riesenberg et al., 2009). As Math5 has been implicated in RGC genesis (Brown et al., 2001; Wang et al., 2001), Neurod1 in photoreceptor (Morrow et al., 1999), Neurod1 with Math3 in AC (Inoue et al., 2002) and Mash1 with Math3 in BC genesis (Tomita et al., 2000), the dependence of these bHLH factors on Pax6 may contribute to the loss of several cell types in Pax6-deficient retinae. However, how Pax6 contributes to HC development has not been studied. Horizontal cells are generated in the first wave of retinogenesis starting around E11 (Hatakeyama and Kageyama, 2004). At this stage, Foxn4 is expressed in mitotic progenitors that give rise to HC/AC common precursors (Li et al., 2004). Although Foxn4 inactivation clearly showed that Foxn4 is essential for HC differentiation, it has been suggested that an additional progenitor-expressed factor, most probably Pax6, may contribute to HC genesis as Foxn4 alone promotes the AC fate only when over-expressed in retinal progenitors (Li et al., 2004). Accordingly, it was previously shown that co-expression of Math3 and Pax6 promotes HC formation (Inoue et al., 2002). However, because HCs develop normally in Math3-null mice (Inoue et al., 2002), other genes may be involved in HC development. In this study we searched for Pax6 downstream-acting transcription factors that might execute this task and identify two transcription factors of the Oc family, Oc1 and Oc2. In *Oc1* and *Oc2* mutants, the number of HCs was dramatically reduced and no HCs developed when both *Oc1* and *Oc2* were inactivated, indicating their essential role in HC genesis. It goes along with the recent studies that reported a decrease of HCs in Oc-deficient retinae (Sapkota et al., 2014; Wu et al., 2013). As Oc1 expression was found to be dependent on Foxn4 expression as well (Wu et al., 2013), and Foxn4 and Pax6 are expressed independently of each other (Li et al., 2004; this study), Pax6 and Foxn4 might cooperate to activate *Oc1* and *Oc2* expression in the retina (Fig. 8). Accordingly, Pax6 or Foxn4 alone do not promote HC genesis when over-expressed in P0 or E17.5 retinal progenitors (Inoue et al., 2002), indicating that none of them is sufficient although both are necessary (Klimova and Kozmik, 2014; Li et al., 2004; Marquardt et al., 2001). Apart from Foxn4 and Pax6, bHLH pancreas-specific transcription factor 1a (Ptf1a) and homeodomain protein Prox1 are required for HC differentiation (Dyer et al., 2003; Fujitani et al., 2006). Ptf1a operates downstream of Foxn4 and is transiently expressed in post-mitotic AC/HC precursors (Fujitani et al., 2006) (Fig. 8). While Ptf1a and Foxn4 specify the HC/AC common precursor and are required for differentiation of both cell types (Fujitani et al., 2006; Li et al., 2004; Nakhai et al., 2007), Prox1, acting downstream of Ptf1a and Foxn4 (Fujitani et al., 2006; Li et al., 2004), is required specifically for acquisition of HC fate (Dyer et al., 2003). Prox1 is the only factor identified to date that is both necessary and sufficient for the HC fate determination (Dyer et al., 2003). Oc1 and Oc2 appear to be dispensable for the initial steps of HC differentiation as the expression of Foxn4, Ptf1a, Prox1 and Lim1 is



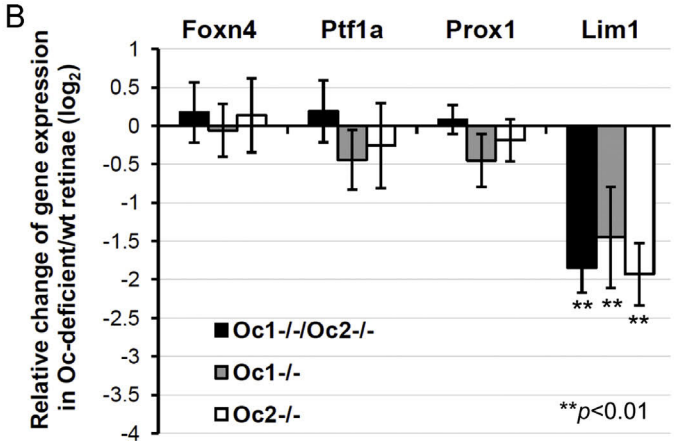


Fig. 6. Horizontal cell differentiation is initiated in Oc1 and Oc2-deficient retinae. (A) Retinal sections of E14.5 (and E16.5) wild-type (*wt*), $Oc1^{-/-}$, $Oc2^{-/-}$ and $Oc1^{-/-}/Oc2^{-/-}$ eyes assessed for the expression of HC-specific factors Foxn4, Ptf1a, Prox1 and Lim1. *In situ* hybridization was used to analyze the mRNA expression of *Foxn4* and immunohistochemistry was used to analyze the protein expression of Ptf1a, Prox1 and Lim1. (B) Quantification of *Foxn4*, *Ptf1a*, *Prox1* and *Lim1* mRNA expression at E14.5 assessed by qRT-PCR. Whole retinae from E14.5 wild-type (*wt*), $Oc1^{-/-}$, $Oc2^{-/-}$ and $Oc1^{-/-}/Oc2^{-/-}$ eyes were dissected, subjected to RNA isolation and processed by qRT-PCR. Error bars indicate standard deviation, *p*-Values are calculated by Student's *t*-test. le, lens; re, retina. Scale bar 200 μm.

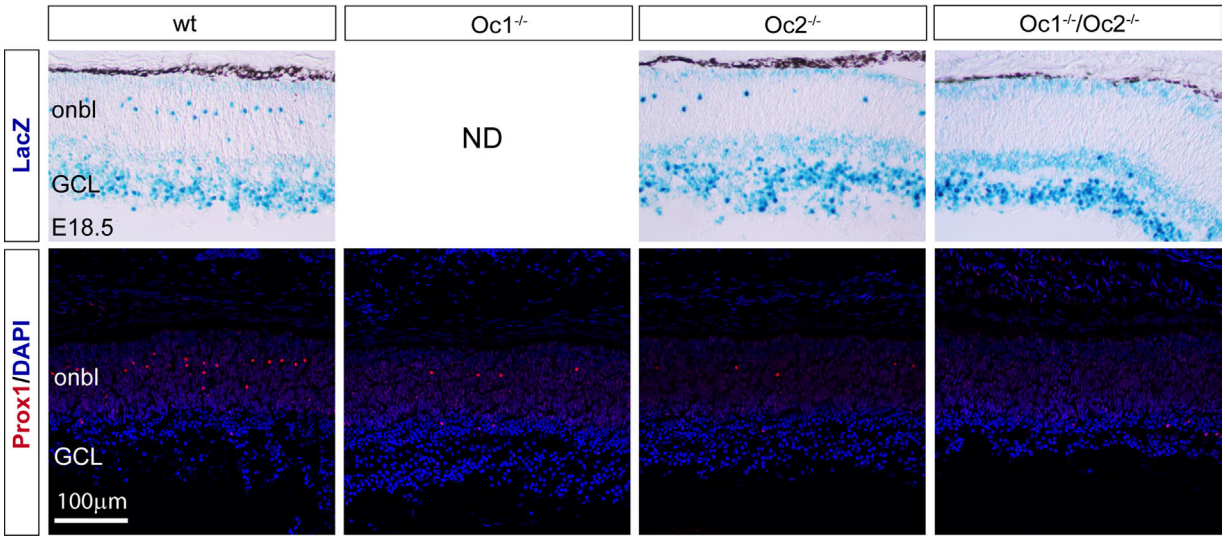


Fig. 7. Oc factors are involved in the maintenance of HCs. Retinal sections of E18.5 *Oc2*$^{+/-}$ (*wt*), *Oc1*$^{-/-}$, *Oc2*$^{-/-}$ and *Oc1*$^{-/-}$/*Oc2*$^{-/-}$ eyes assessed for the expression of HC-specific factors Oc2 and Prox1. As *Oc2* knockout mice were generated by the insertion of *LacZ* into the *Oc2* coding sequence (Clotman et al., 2005), β-galactosidase expression was used to trace the expression of *Oc2*. β-galactosidase and Prox1 staining shows that HCs are lost already at E18.5.

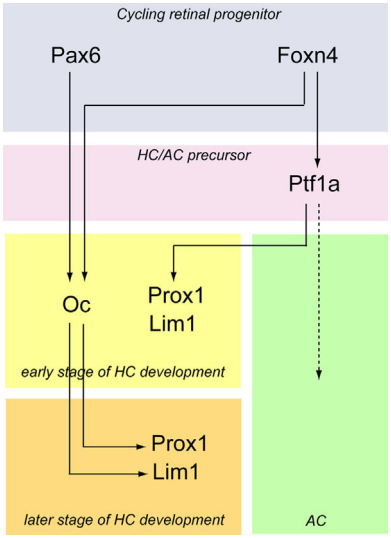


Fig. 8. Schematic representation of the Pax6, Oc1 and Oc2 role in HC development. Pax6 and Foxn4 are co-expressed in cycling retinal progenitor cells that give rise to both HCs and ACs (Li et al., 2004). In the common progenitor of HCs and ACs, Foxn4 stimulates expression of Ptf1a (Fujitani et al., 2006), which is required for differentiation of both cell types (Fujitani et al., 2006; Nakhai et al., 2007). To specify the HC fate, Pax6 and Foxn4 stimulate expression of Oc1/Oc2 in HC precursors (this study; Wu et al., 2013). At early stages of HC development, the expression of Prox1 and Lim1 is not dependent on Oc expression. At later stages of HC development, Oc factors are required to maintain the HC identity most likely through the regulation of Lim1 and Prox1 expression. The sequence of events leading to differentiation of HCs is indicated by solid arrows, events leading to differentiation of ACs with dashed arrow.

initiated in Oc-deficient retinae. Recent studies have indicated that Prox1 and Lim1 expression is not maintained upon Oc inactivation, suggesting the model in which Oc factors operate upstream of Prox1 to direct HC differentiation (Sapkota et al., 2014; Wu et al., 2013). However, we used three independent approaches to analyze the expression of Prox1 in *Oc*-deficient retinae and found that Prox1 (as well as Lim1) expression is initiated and Prox1^{+}/Lim1^{+} HCs are initially generated. Consistently, RNA-seq data presented by Sapkota et al. (2014) indicated only very slight decrease of Prox1 expression in Oc-deficient retinae.

Studies of the Oc role in different parts of the nervous system indicate that Oc factors are responsible for the maintenance of specific cell fate and survival rather than for the initial steps of differentiation (Espana and Clotman, 2012a, 2012b; Stam et al., 2012). A similar phenomenon can be observed in the retina as well. The expression of the main determinants of HC fate is initiated, indicating that the differentiation program of HCs is initiated properly but not maintained in the absence of *Oc1* and *Oc2*. As Lim1 and Prox1 expression is lost soon after HCs have been generated, the maintenance of HCs may require the continuous Lim1 and Prox1 expression at later stages of HC development (Fig. 8). Accordingly, Oc1 misexpression in postnatal mouse retina is sufficient to induce Lim1 expression (Emerson et al., 2013). The Pax6-regulated Oc pathway might then represent the branch in HC GRN, ensuring rather the maintenance of HC fate than the initial cell fate determination (Fig. 8). However, considering that HCs represent only approximately 0.2% of all retinal cells in the mouse (Ajioka et al., 2007), it is complicated to determine whether these cells died or adopted an alternative retinal cell fate. Further studies need to be executed to answer this issue.

Apart from HCs, strong expression of Oc1 and Oc2 was observed in developing RGCs (Wu et al., 2012). RGCs are the first retinal cell type to differentiate in the mammalian retina (Cepko et al., 1996; Young, 1985) and their differentiation is governed by a well-defined GRN of three transcription factors; Math5, Brn3b and Isl1. Whereas Math5 keeps RPCs competent to generate RGCs, Brn3b and Isl1 function downstream of Math5 and activate genes essential for RGC differentiation (Mu et al., 2008; Pan et al., 2008). As Oc1 and Oc2 expression was found to overlap with Math5 as well as with Brn3b and Isl1, the Oc role in establishing RGC fate was suggested (Wu et al., 2012). Since Oc1 and Oc2 expression appeared to be independent of Isl1, Brn3b and Math5, it was assumed that Oc regulate RGC genesis independently of these factors and represent a novel pathway in the RGC GRN (Wu et al., 2012). Accordingly, our analysis showed that *Oc1* and *Oc2* expression is dependent on Pax6 expression, integrating Oc1 and Oc2 downstream of Pax6, to the parallel with that of Math5 in RGC GRN. Interestingly, Oc inactivation seems to have only a negligible impact on RGC differentiation in general. On the other hand, a small subset of RGCs (~10%), Tbr2^{+} RGCs, is severally affected (Sapkota et al., 2014; this study). It is represented by RGC types that send axonal projections to non-image forming areas of brain,

such as Opn4/melanopsin-expressing photosensitive RGCs (ipRGCs) (Mao et al., 2008, 2014; Sweeney et al., 2014). Oc factors are then likely to be involved specifically in generation of this RGC subpopulation.

Until now, Oc expression has been observed in differentiating HCs, RGCs and cone photoreceptors (Emerson et al., 2013; Wu et al., 2012). However, analysis of other retinal cell types in Oc-deficient retinae indicates Oc's role in differentiation of bipolar cells, amacrine cells and Müller glia cells as well (Sapkota et al., 2014; this study). Since we do not have detailed information about Oc expression in such retinal cell types, we cannot distinguish whether the effect of Oc loss is caused by cell-autonomous or non-cell-autonomous mechanism. As Oc1 and Oc2 expression in RPCs has been previously reported (Emerson et al., 2013; Wu et al., 2012), it raises the question whether Oc factors could be transiently expressed in progenitors of retinal cell types other than HCs, RGCs and cone photoreceptors. More comprehensive analysis combined with the tracing of Oc-expressing cells would help to resolve this issue.

In different systems studied, Oc1 and Oc2 show functional redundancy as they are expressed in a partially overlapping pattern and share biochemical activities (Clotman et al., 2005; Espana and Clotman, 2012a, b; Jacquemin et al., 1999; Margagliotti et al., 2007; Vanhorenbeeck et al., 2002, 2007). In neuronal populations, including A13 neurons, Locus Coereleus neurons and Renshaw cells, inactivation of *Oc1* or *Oc2* alone results in no or mild defective phenotype only, when compared with compound *Oc1*/*Oc2* mutants (Espana and Clotman, 2012a, 2012b; Stam et al., 2012). Interestingly, in the retina, the degree of redundancy appears lower as inactivation of a single *Oc* gene leads to dramatic reduction of HCs. Although the phenotypes of *Oc1* and *Oc2* share general characteristics and Oc1 and Oc2 probably exert overlapping functions, we cannot formally exclude the possibility that Oc1 and Oc2 may display distinct properties during the HC development. Further, more detailed analysis and comparison of Oc1 and Oc2 target genes that mediate HC genesis might resolve this issue.

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While this article was under review the manuscript Sapkota et al. was published describing the analysis of Oc1/Oc2 double K.O. mice. To address some discrepancies in our initial submission immunohistochemical stainings at P18 were performed for the following markers: Tbr2, Rxrγ, Chx10 and Lhx2.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.02.023.

References

Ajioka, I., Martins, R.A., Bayazitov, I.T., Donovan, S., Johnson, D.A., Frase, S., Cicero, S. A., Boyd, K., Zakharenko, S.S., Dyer, M.A., 2007. Differentiated horizontal interneurons clonally expand to form metastatic retinoblastoma in mice. Cell 131, 378–390.

Audouard, E., Schakman, O., Ginion, A., Bertrand, L., Gailly, P., Clotman, F., 2013. The Onecut transcription factor HNF-6 contributes to proper reorganization of Purkinje cells during postnatal cerebellum development. Mol. Cell. Neurosci. 56, 159–168.

Bassett, E.A., Korol, A., Deschamps, P.A., Buettner, R., Wallace, V.A., Williams, T., West-Mays, J.A., 2012. Overlapping expression patterns and redundant roles for AP-2 transcription factors in the developing mammalian retina. Dev. Dyn. 241, 814–829.

Bassett, E.A., Pontoriero, G.F., Feng, W., Marquardt, T., Fini, M.E., Williams, T., West-Mays, J.A., 2007. Conditional deletion of activating protein 2alpha (AP-2alpha) in the developing retina demonstrates non-cell-autonomous roles for AP-2alpha in optic cup development. Mol. Cell. Biol. 27, 7497–7510.

Brown, N.L., Kanekar, S., Vetter, M.L., Tucker, P.K., Gemza, D.L., Glaser, T., 1998. Math5 encodes a murine basic helix-loop-helix transcription factor expressed during early stages of retinal neurogenesis. Development 125, 4821–4833.

Brown, N.L., Patel, S., Brzezinski, J., Glaser, T., 2001. Math5 is required for retinal ganglion cell and optic nerve formation. Development 128, 2497–2508.

Cepko, C.L., 1999. The roles of intrinsic and extrinsic cues and bHLH genes in the determination of retinal cell fates. Curr. Opin. Neurobiol. 9, 37–46.

Cepko, C.L., Austin, C.P., Yang, X., Alexiades, M., Ezzeddine, D., 1996. Cell fate determination in the vertebrate retina. Proc. Natl. Acad. Sci. USA 93, 589–595.

Clotman, F., Jacquemin, P., Plumb-Rudewiez, N., Pierreux, C.E., Van der Smissen, P., Dietz, H.C., Courtoy, P.J., Rousseau, G.G., Lemaigre, F.P., 2005. Control of liver cell fate decision by a gradient of TGF beta signaling modulated by Onecut transcription factors. Genes Dev. 19, 1849–1854.

Clotman, F., Lannoy, V.J., Reber, M., Cereghini, S., Cassiman, D., Jacquemin, P., Roskams, T., Rousseau, G.G., Lemaigre, F.P., 2002. The onecut transcription factor HNF6 is required for normal development of the biliary tract. Development 129, 1819–1828.

de Melo, J., Peng, G.H., Chen, S., Blackshaw, S., 2011. The Spalt family transcription factor Sall3 regulates the development of cone photoreceptors and retinal horizontal interneurons. Development 138, 2325–2336.

de Melo, J., Qiu, X., Du, G., Cristante, L., Eisenstat, D.D., 2003. Dlx1, Dlx2, Pax6, Brn3b, and Chx10 homeobox gene expression defines the retinal ganglion and inner nuclear layers of the developing and adult mouse retina. J. Comp. Neurol. 461, 187–204.

Dusing, M.R., Maier, E.A., Aronow, B.J., Wiginton, D.A., 2010. Onecut-2 knockout mice fail to thrive during early postnatal period and have altered patterns of gene expression in small intestine. Physiol. Genomics 42, 115–125.

Dyer, M.A., Livesey, F.J., Cepko, C.L., Oliver, G., 2003. Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina. Nat. Genet. 34, 53–58.

Emerson, M.M., Surzenko, N., Goetz, J.J., Trimarchi, J., Cepko, C.L., 2013. Otx2 and Onecut1 promote the fates of cone photoreceptors and horizontal cells and repress rod photoreceptors. Dev. Cell 26, 59–72.

Espana, A., Clotman, F., 2012a. Onecut factors control development of the *Locus Coeruleus* and of the mesencephalic trigeminal nucleus. Mol. Cell. Neurosci. 50, 93–102.

Espana, A., Clotman, F., 2012b. Onecut transcription factors are required for the second phase of development of the A13 dopaminergic nucleus in the mouse. J. Comp. Neurol. 520, 1424–1441.

Fujimura, N., Taketo, M.M., Mori, M., Korinek, V., Kozmik, Z., 2009. Spatial and temporal regulation of Wnt/beta-catenin signaling is essential for development of the retinal pigment epithelium. Dev. Biol. 334, 31–45.

Fujitani, Y., Fujitani, S., Luo, H., Qiu, F., Burlison, J., Long, Q., Kawaguchi, Y., Edlund, H., MacDonald, R.J., Furukawa, T., Fujikado, T., Magnuson, M.A., Xiang, M., Wright, C.V., 2006. Ptf1a determines horizontal and amacrine cell fates during mouse retinal development. Development 133, 4439–4450.

Hatakeyama, J., Kageyama, R., 2004. Retinal cell fate determination and bHLH factors. Semin. Cell Dev. Biol. 15, 83–89.

Haworth, K.E., Latinkic, B., 2009. Expression of Xenopus tropicalis HNF6/Onecut-1. Int. J. Dev. Biol. 53, 159–162.

Hodge, L.K., Klassen, M.P., Han, B.X., Yiu, G., Hurrell, J., Howell, A., Rousseau, G., Lemaigre, F., Tessier-Lavigne, M., Wang, F., 2007. Retrograde BMP signaling regulates trigeminal sensory neuron identities and the formation of precise face maps. Neuron 55, 572–586.

Holt, C.E., Bertsch, T.W., Ellis, H.M., Harris, W.A., 1988. Cellular determination in the Xenopus retina is independent of lineage and birth date. Neuron 1, 15–26.

Chakrabarty, K., Von Oerthel, L., Hellemons, A., Clotman, F., Espana, A., Groot Koerkamp, M., Holstege, F.C., Pasterkamp, R.J., Smidt, M.P., 2012. Genome wide expression profiling of the mesodiencephalic region identifies novel factors involved in early and late dopaminergic development. Biol. Open 1, 693–704.

Inoue, T., Hojo, M., Bessho, Y., Tano, Y., Lee, J.E., Kageyama, R., 2002. Math3 and NeuroD regulate amacrine cell fate specification in the retina. Development 129, 831–842.

Jacquemin, P., Durviaux, S.M., Jensen, J., Godfraind, C., Gradwohl, G., Guillemot, F., Madsen, O.D., Carmeliet, P., Dewerchin, M., Collen, D., Rousseau, G.G., Lemaigre, F.P., 2000. Transcription factor hepatocyte nuclear factor 6 regulates pancreatic endocrine cell differentiation and controls expression of the proendocrine gene ngn3. Mol. Cell. Biol. 20, 4445–4454.

Jacquemin, P., Lannoy, V.J., Rousseau, G.G., Lemaigre, F.P., 1999. OC-2, a novel mammalian member of the ONECUT class of homeodomain transcription factors whose function in liver partially overlaps with that of hepatocyte nuclear factor-6. J. Biol. Chem. 274, 2665–2671.

Jacquemin, P., Lemaigre, F.P., Rousseau, G.G., 2003. The Onecut transcription factor HNF-6 (OC-1) is required for timely specification of the pancreas and acts upstream of Pdx-1 in the specification cascade. Dev. Biol. 258, 105–116.

Klimova, L., Kozmik, Z., 2014. Stage-dependent requirement of neuroretinal Pax6 for lens and retina development. Development 141, 1292–1302.
Klimova, L., Lachova, J., Machon, O., Sedlacek, R., Kozmik, Z., 2013. Generation of mRx-Cre transgenic mouse line for efficient conditional gene deletion in early retinal progenitors. PLoS One 8, e63029.
Kozmik, Z., 2005. Pax genes in eye development and evolution. Curr. Opin. Genet. Dev. 15, 430–438.
Kozmik, Z., Czerny, T., Busslinger, M., 1997. Alternatively spliced insertions in the paired domain restrict the DNA sequence specificity of Pax6 and Pax8. EMBO J. 16, 6793–6803.
Lannoy, V.J., Burglin, T.R., Rousseau, G.G., Lemaigre, F.P., 1998. Isoforms of hepatocyte nuclear factor-6 differ in DNA-binding properties, contain a bifunctional homeodomain, and define the new ONECUT class of homeodomain proteins. J. Biol. Chem. 273, 13552–13562.
Li, S., Mo, Z., Yang, X., Price, S.M., Shen, M.M., Xiang, M., 2004. Foxn4 controls the genesis of amacrine and horizontal cells by retinal progenitors. Neuron 43, 795–807.
Lillien, L., 1995. Changes in retinal cell fate induced by overexpression of EGF receptor. Nature 377, 158–162.
Liu, H., Kim, S.Y., Fu, Y., Wu, X., Ng, L., Swaroop, A., Forrest, D., 2013. An isoform of retinoid-related orphan receptor beta directs differentiation of retinal amacrine and horizontal interneurons. Nat. Commun. 4, 1813.
Liu, W., Wang, J.H., Xiang, M., 2000. Specific expression of the LIM/homeodomain protein Lim-1 in horizontal cells during retinogenesis. Dev. Dyn. 217, 320–325.
Mao, C.A., Kiyama, T., Pan, P., Furuta, Y., Hadjantonakis, A.K., Klein, W.H., 2008. Eomesodermin, a target gene of Pou4f2, is required for retinal ganglion cell and optic nerve development in the mouse. Development 135, 271–280.
Mao, C.A., Li, H., Zhang, Z., Kiyama, T., Panda, S., Hattar, S., Ribelayga, C.P., Mills, S.L., Wang, S.W., 2014. T-box transcription regulator Tbr2 is essential for the formation and maintenance of Opn4/melanopsin-expressing intrinsically photosensitive retinal ganglion cells. J. Neurosci. 34, 13083–13095.
Margagliotti, S., Clotman, F., Pierreux, C.E., Beaudry, J.B., Jacquemin, P., Rousseau, G. G., Lemaigre, F.P., 2007. The Onecut transcription factors HNF-6/OC-1 and OC-2 regulate early liver expansion by controlling hepatoblast migration. Dev. Biol. 311, 579–589.
Marquardt, T., Ashery-Padan, R., Andrejewski, N., Scardigli, R., Guillemot, F., Gruss, P., 2001. Pax6 is required for the multipotent state of retinal progenitor cells. Cell 105, 43–55.
Morrow, E.M., Furukawa, T., Lee, J.E., Cepko, C.L., 1999. NeuroD regulates multiple functions in the developing neural retina in rodent. Development 126, 23–36.
Mu, X., Fu, X., Beremand, P.D., Thomas, T.L., Klein, W.H., 2008. Gene regulation logic in retinal ganglion cell development: Isl1 defines a critical branch distinct from but overlapping with Pou4f2. Proc. Natl. Acad. Sci. USA 105, 6942–6947.
Nakhai, H., Sel, S., Favor, J., Mendoza-Torres, L., Paulsen, F., Duncker, G.I., Schmid, R. M., 2007. Ptf1a is essential for the differentiation of GABAergic and glycinergic amacrine cells and horizontal cells in the mouse retina. Development 134, 1151–1160.
Oron-Karni, V., Farhy, C., Elgart, M., Marquardt, T., Remizova, L., Yaron, O., Xie, Q., Cvekl, A., Ashery-Padan, R., 2008. Dual requirement for Pax6 in retinal progenitor cells. Development 135, 4037–4047.
Pan, L., Deng, M., Xie, X., Gan, L., 2008. ISL1 and BRN3B co-regulate the differentiation of murine retinal ganglion cells. Development 135, 1981–1990.
Perron, M., Harris, W.A., 2000. Determination of vertebrate retinal progenitor cell fate by the Notch pathway and basic helix-loop-helix transcription factors. Cell. Mol. Life Sci. 57, 215–223.
Pierreux, C.E., Poll, A.V., Kemp, C.R., Clotman, F., Maestro, M.A., Cordi, S., Ferrer, J., Leyns, L., Rousseau, G.G., Lemaigre, F.P., 2006. The transcription factor hepatocyte nuclear factor-6 controls the development of pancreatic ducts in the mouse. Gastroenterology 130, 532–541.
Poche, R.A., Kwan, K.M., Raven, M.A., Furuta, Y., Reese, B.E., Behringer, R.R., 2007. Lim1 is essential for the correct laminar positioning of retinal horizontal cells. J. Neurosci. 27, 14099–14107.
Riesenberg, A.N., Le, T.T., Willardsen, M.I., Blackburn, D.C., Vetter, M.L., Brown, N.L., 2009. Pax6 regulation of Math5 during mouse retinal neurogenesis. Genesis 47, 175–187.
Roberts, M.R., Hendrickson, A., McGuire, C.R., Reh, T.A., 2005. Retinoid X receptor (gamma) is necessary to establish the S-opsin gradient in cone photoreceptors of the developing mouse retina. Investig. Ophthalmol. Vis. Sci. 46, 2897–2904.
Roesch, K., Jadhav, A.P., Trimarchi, J.M., Stadler, M.B., Roska, B., Sun, B.B., Cepko, C.L., 2008. The transcriptome of retinal Muller glial cells. J. Comp. Neurol. 509, 225–238.
Roy, A., Francius, C., Rousso, D.L., Seuntjens, E., Debruyn, J., Luxenhofer, G., Huber, A.B., Huylebroeck, D., Novitch, B.G., Clotman, F., 2012. Onecut transcription factors act upstream of Isl1 to regulate spinal motoneuron diversification. Development 139, 3109–3119.
Sandelin, A., Alkema, W., Engstrom, P., Wasserman, W.W., Lenhard, B., 2004. JASPAR: an open-access database for eukaryotic transcription factor binding profiles. Nucleic Acids Res. 32, D91–94.
Sapkota, D., Chintala, H., Wu, F., Fliesler, S.J., Hu, Z., Mu, X., 2014. Onecut1 and Onecut2 redundantly regulate early retinal cell fates during development. Proc. Natl. Acad. Sci. USA 111, E4086–4095.
Schwarz, M., Cecconi, F., Bernier, G., Andrejewski, N., Kammandel, B., Wagner, M., Gruss, P., 2000. Spatial specification of mammalian eye territories by reciprocal transcriptional repression of Pax2 and Pax6. Development 127, 4325–4334.
Stam, F.J., Hendricks, T.J., Zhang, J., Geiman, E.J., Francius, C., Labosky, P.A., Clotman, F., Goulding, M., 2012. Renshaw cell interneuron specialization is controlled by a temporally restricted transcription factor program. Development 139, 179–190.
Sweeney, N.T., Tierney, H., Feldheim, D.A., 2014. Tbr2 is required to generate a neural circuit mediating the pupillary light reflex. J. Neurosci. 34, 5447–5453.
Tomita, K., Ishibashi, M., Nakahara, K., Ang, S.L., Nakanishi, S., Guillemot, F., Kageyama, R., 1996. Mammalian hairy and Enhancer of split homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. Neuron 16, 723–734.
Tomita, K., Moriyoshi, K., Nakanishi, S., Guillemot, F., Kageyama, R., 2000. Mammalian achaete-scute and atonal homologs regulate neuronal *versus* glial fate determination in the central nervous system. EMBO J. 19, 5460–5472.
Turner, D.L., Cepko, C.L., 1987. A common progenitor for neurons and glia persists in rat retina late in development. Nature 328, 131–136.
Turner, D.L., Snyder, E.Y., Cepko, C.L., 1990. Lineage-independent determination of cell type in the embryonic mouse retina. Neuron 4, 833–845.
Vanhorenbeeck, V., Jacquemin, P., Lemaigre, F.P., Rousseau, G.G., 2002. OC-3, a novel mammalian member of the ONECUT class of transcription factors. Biochem. Biophys. Res. Commun. 292, 848–854.
Vanhorenbeeck, V., Jenny, M., Cornut, J.F., Gradwohl, G., Lemaigre, F.P., Rousseau, G. G., Jacquemin, P., 2007. Role of the Onecut transcription factors in pancreas morphogenesis and in pancreatic and enteric endocrine differentiation. Dev. Biol. 305, 685–694.
Walther, C., Gruss, P., 1991. Pax-6, a murine paired box gene, is expressed in the developing CNS. Development 113, 1435–1449.
Wang, S.W., Kim, B.S., Ding, K., Wang, H., Sun, D., Johnson, R.L., Klein, W.H., Gan, L., 2001. Requirement for math5 in the development of retinal ganglion cells. Genes Dev. 15, 24–29.
Wu, F., Li, R., Umino, Y., Kaczynski, T.J., Sapkota, D., Li, S., Xiang, M., Fliesler, S.J., Sherry, D.M., Gannon, M., Solessio, E., Mu, X., 2013. Onecut1 is essential for horizontal cell genesis and retinal integrity. J. Neurosci. 33, 13053–13065 13065a.
Wu, F., Sapkota, D., Li, R., Mu, X., 2012. Onecut 1 and Onecut 2 are potential regulators of mouse retinal development. J. Comp. Neurol. 520, 952–969.
Xiang, M., Zhou, L., Macke, J.P., Yoshioka, T., Hendry, S.H., Eddy, R.L., Shows, T.B., Nathans, J., 1995. The Brn-3 family of POU-domain factors: primary structure, binding specificity, and expression in subsets of retinal ganglion cells and somatosensory neurons. J. Neurosci. 15, 4762–4785.
Young, R.W., 1985. Cell differentiation in the retina of the mouse. Anat. Rec. 212, 199–205.
Zhang, H., Ables, E.T., Pope, C.F., Washington, M.K., Hipkens, S., Means, A.L., Path, G., Seufert, J., Costa, R.H., Leiter, A.B., Magnuson, M.A., Gannon, M., 2009. Multiple, temporal-specific roles for HNF6 in pancreatic endocrine and ductal differentiation. Mech. Dev. 126, 958–973.